RATE OF NITRIC OXIDE FORMATION FROM NITRITE AS AFFECTED BY CHLORIDE ION CONCENTRATION

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ABSTRACT

The role of chloride ions in nitrite reactions was assessed by measuring the rates of nitric oxide formation in a simplified system consisting of sodium nitrite, chloride, ascorbate, horse myoglobin and bovine serum albumin in acetate buffer. The addition of chloride ions accelerated the formation of nitric oxide, the rate increasing linearly with chloride ion concentration. The chloride ion effect was observed to be greater with increasingly acidic conditions. With exposure to air nitric oxide myoglobin converted to metmyoglobin, but remained stable when held under nitrogen.

INTRODUCTION

Cured meat products are manufactured in a variety of ways but two common ingredients that are essential for the development of expected product characteristics are sodium chloride and sodium nitrite. Each of these has specific functions which have been largely assumed to be independent of each other; sodium chloride contributing to the flavor and reduced a_w, sodium nitrite creating cured color and greater flavor stability. Both compounds contribute to microbial control through a number of mechanisms. The functions of nitrite have been of particular interest due to the complexity of reaction sequences leading to the end effects (Cassens *et al.* 1979). The effect of chloride ions on nitrite reactions in meat is not clear but there is evidence that interactions do occur. Roberts and Ingram (1973) demonstrated a three-way interaction between pH, chloride and nitrite in the inhibition of growth of *C. botulinum*. While the inhibitory effect of chloride and nitrite was greater than that of either alone, the

interaction terms were negative, that is, the combined effect was less than the sum of the individual inhibitions (Roberts *et al.* 1976). This implies that chloride and nitrite ions were forming a compound that was less effective than nitrous acid alone. Chloride ions increased apparent residual nitrite values as determined by the Griess method when sulfanilic acid was used for the Griess reagent (Hildrum 1979), but there was no chloride ion effect when sulfanilamide was employed (Fox *et al.* 1981). These differential effects may explain some of the contradictions in the literature when presence of sodium chloride is reported to result in increased levels of nitrite (Fujimaki *et al.* 1975), to have no effect on nitrite (Olsman and Krol 1972) or to decrease levels of residual nitrite (Lee and Cassens 1980). In addition to the chloride effect noted in Griess reagent studies, there have been suggestions that the presence of chloride ions suppressed formation of nitrosopyrrolidine and nitrosoproline (Theiler *et al.* 1981; Hildrum *et al.* 1975) in meat or model systems. In these chloride/nitrite interactions the effect of chloride ions was sometimes positive, sometimes negative.

Cured color formation is probably the best characterized nitrite reaction, having been extensively studied (Fox and Thomson 1963; Fox and Ackerman 1968; MacDougall *et al.*; Lin and Sebranek 1979). Cured meat color development follows the nitric oxide myoglobin (NOMb) formation after the formation of nitric oxide (NO) from one or more of several possible reactive intermediates (Sebranek and Fox 1986). One of the possible reaction intermediates that could contribute to cured meat color is nitrosyl chloride (NOCl), a nitrosating intermediate known to be formed under very acidic conditions (Challis and Shuker 1979; Turney and Wright 1959). It has been occasionally observed that meat curing mixtures formulated with reduced salt content require somewhat longer curing time to develop a cured color than those with higher salt concentrations. If nitrosyl chloride was a contributing nitrosating agent, reducing the chloride ion concentration would result in a longer curing time. This study was undertaken to evaluate the potential effects of chloride ion concentration on nitrite reactions under conditions similar to those occurring in the preparation of cured meat systems.

MATERIALS AND METHODS

The system chosen for this study consisted of an acetate buffer solution containing bovine serum albumin, metmyoglobin, sodium ascorbate, sodium nitrite and sodium chloride. The nitric oxide reaction rate was followed by periodically scanning the visible spectra from 470-560 nm of the reacting solutions, since the major absorption bands of metmyoglobin and nitric oxide myoglobin are at 505

nm and 545 nm, respectively. Multiple wavelength analysis was used to determine the amounts of the two pigment forms existing in the solution at any given time.

The preparation of the reaction mixtures required care to remove any residual or dissolved oxygen. Nitrogen was bubbled through all water to be used for solutions at least 24 h prior to use. The presence of oxygen tended to delay or slow the reaction as measured, probably by combining with some of the generated nitric oxide as it was formed. The acetate buffer was prepared by adjusting a 0.1 M acetate solution to the target pH (5.4, 5.8 or 6.2). Sodium chloride representing 0.05, 1, 2 and 4% of the final solution (weight/volume) was weighed into individual Erlenmeyer flasks after which the flasks were placed in a glovebox under a constant stream of nitrogen. Metmyoglobin (horse heart, Sigma Chemical Co., St. Louis, Mo.) was dissolved in acetate buffer at a concentration of 0.3 mM along with 2% (weight/volume) bovine serum albumin (BSA). Twenty-three milliliters of this solution were pipetted to each of the flasks with sodium chloride and the salt was allowed to dissolve. Since increasing concentrations of sodium chloride decreased the pH of the solution slightly, the contents of each flask were then individually adjusted back to the pH of the 0% sodium chloride sample using 0.1 N NaOH. Following the pH adjustment, stock solutions of sodium nitrite and sodium ascorbate were used to pipette 1 mL of nitrite solution to each flask followed by 1 mL of ascorbate. Concentrations of the stock solutions ranged from 5.75 mM to 56.5 mM for nitrite and from 0.78 mM to 78.3 mM for ascorbate. These stock solutions resulted in final concentrations listed in Table 1. The flasks were immediately swirled and a portion of each was transferred to cuvettes for scanning on a Cary Model 14 spectrophotometer. Cuvettes were transferred from the glovebox to the spectrophotometer sample compartment which was also flushed by a constant stream of nitrogen. The sample compartment accommodated all 5 samples in a rotating cuvette holder, thus subsequent transfer of cuvettes was not required.

Comparisons of reaction rates in air were conducted on samples prepared in the same fashion except that solutions were not bubbled with nitrogen prior to use and flasks and cuvettes were held in the ambient atmosphere rather than the glovebox. Three replications of each combination of reactants were conducted. Nitrite concentrations examined included 2.26, 1.80, 1.36, 0.93, 0.46 and 0.23 mM (15, 30, 60, 90, 120 and 150 ppm in solution, respectively). Ascorbic concentrations included 3.13, 1.28, 0.64 and 0.31 mM (55, 110, 220 and 550 ppm in solution, respectively). This rate of reaction (nitric oxide myoglobin formation) as influenced by sodium chloride concentration was measured at each combination of nitrite/ascorbate concentrations to assess the potential effect of chloride on nitric oxide formation. Data were analyzed according to Snedecor and Cochran (1967). Coefficient of variation was used to express mean variability.

TABLE 1.
RATE OF FORMATION OF NO[1]

mM Nitrite	mM Ascorbate	%NaCl 0 pH	0 k[2]	0.5 pH	0.5 k	1 pH	1 k	2 pH	2 k	4 pH	4 k
2.25	3.13	5.45	5.00	5.40	5.60	5.37	6.60	5.33	7.30	5.30	8.30
		5.76	2.80	5.72	3.15	5.69	3.15	5.66	3.50	5.62	4.38
		6.32	1.46	6.28	1.46	6.26	1.56	6.23	1.56	6.19	1.66
1.80	3.13	5.44	5.00	5.40	5.30	5.38	5.60	5.33	6.60	5.28	8.60
		5.76	1.82	5.73	1.98	5.70	2.15	5.67	2.15	5.63	2.30
		6.29	1.20	6.24	1.20	6.22	1.20	6.19	1.09	6.15	1.09
1.36	3.13	5.46	4.00	5.41	4.00	5.38	4.60	5.34	5.60	5.28	6.60
		5.75	3.30	5.72	3.30	5.68	3.30	5.66	4.13	5.61	4.13
		6.29	1.10	6.23	1.04	6.22	1.04	6.17	1.04	6.14	0.99
0.93	3.13	5.46	2.60	5.41	2.80	5.38	3.00	5.33	3.30	5.28	4.50
		5.75	1.98	5.71	2.48	5.68	2.81	5.65	2.81	5.61	2.81
		6.29	1.07	6.24	1.07	6.23	1.07	6.20	1.05	6.16	1.10
0.46	3.13	5.46	2.50	5.41	2.50	5.38	2.80	5.33	3.10	5.29	4.00
		5.75	1.10	5.72	1.10	5.68	1.10	5.66	1.38	5.61	1.65
		6.30	0.94	6.25	0.94	6.23	0.94	6.20	0.88	6.16	0.88
0.23	3.13	5.43	1.50	5.40	1.67	5.38	1.73	5.33	2.09	5.27	2.53
		5.76	1.20	5.73	1.20	5.70	1.20	5.67	1.20	5.63	1.56
		6.26	0.55	6.21	0.66	6.18	0.44	6.15	0.41	6.11	0.41
2.26	1.28	5.45	3.30	5.40	3.85	5.37	3.85	5.33	4.40	5.28	6.60
		5.77	1.87	5.73	2.20	5.71	2.59	5.68	2.97	5.64	3.47
		6.26	1.28	6.28	1.17	6.26	1.17	6.22	1.28	6.18	1.34
2.26	0.64	5.47	1.65	5.42	1.98	5.40	2.53	5.34	3.63	5.30	5.50
		5.75	1.09	5.72	1.35	5.69	1.51	5.66	2.08	5.62	3.12
		6.26	0.93	6.22	0.82	6.19	0.76	6.15	0.82	6.11	0.93
2.26	0.31	5.48	1.76	5.46	1.87	5.43	2.42	5.39	2.86	5.37	3.85
		5.76	0.78	5.72	0.88	5.70	0.88	5.67	1.04	5.62	1.61
		6.26	0.58	6.21	0.53	6.19	0.53	6.15	0.58	6.11	0.58

[1]Pooled coefficient of variation (C) = 12.3%

[2]k_o = mM · min^{-1} X 10^{-3}

RESULTS AND DISCUSSION

The chloride-ion catalyzed reaction

The simplified system (albumin, metmyoglobin, ascorbate, nitrite and chloride) was chosen because nitrite is likely to be involved with several reaction sequences in a meat system. Serum albumin was utilized to provide a uniform protein environment with less variation than meat proteins. Preliminary studies without albumin showed no significant effect of albumin on reaction rates; thus the treatment with albumin and no sodium chloride was used as a control to compare the effects of added chloride ions.

Metmyoglobin provided a good starting point for reaction rate measurements because the initial reaction when curing salts are added to meat in the presence of oxygen is the oxidation of myoglobin to the ferric (met) form. The oxidized pigment then is the starting form of the pigment for the pink cured color reaction. Preliminary work for this study utilized spectral scans of known pigment solutions containing metmyglobin, nitric oxide myoglobin and nitric oxide metmyoglobin to confirm that only nitric oxide myoglobin was being formed in measureable amounts from metmyoglobin in the system used.

The results expressed as zero order rate constants (k_0=mM $min^{-1} \times 10^{-3}$) are shown in Table 1. Each point is the mean value of three determinations. The results show that increasing the chloride concentration at pH 5.8 or less increased the rate constant for nitric oxide formation, and that the effect was lower at low concentrations of ascorbate ion (Fig. 1). For example, the slope of the curve at pH 5.4 (open squares) was lower with 0.31 mM ascorbate than it was with 3.13 mM ascorbate (solid squares). The hydrogen ion concentration also affected the chloride-ion catalyzed reaction because, as can be seen in Fig. 1, the slopes of the curves at either ascorbate concentration decreased at higher pH value. At pH 6.2 there was no chloride-ion catalyzed reaction. Since hydrochloric acid is completely ionized over this pH range, it appears that nitrous acid was required for the chloride-ion catalyzed reaction, through the formation of nitrosylchloride from nitrous acid and chloride (Turney and Wright 1959; Stedman 1979). Even though nitrosylchloride is a much more powerful nitrosating species than nitrous acid, the total contribution of nitrosylchloride to the overall rate was relatively small. Because the conditions under which it was formed were unfavorable (very low nitrous acid concentration), the concentration of nitrosylchloride was also very low, accounting for the small contribution of the chloride-ion catalyzed reaction to the overall reaction of nitrite ions.

The Nitrous Acid (Noncatalyzed) Reaction

In contrast, the direct reaction of nitrite ions and ascorbate ions was affected to a much less degree in this study than was the chloride-ion catalyzed reaction.

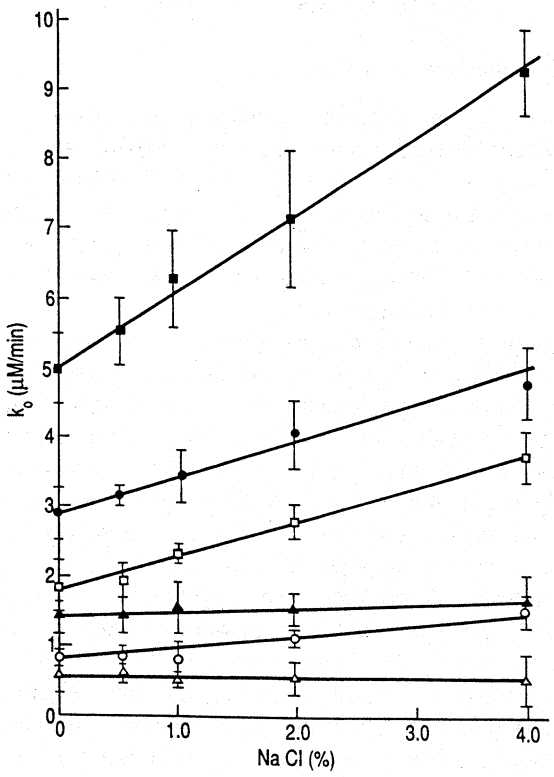

FIG. 1. ZERO ORDER RATE CONSTANTS FOR THE FORMATION OF NITRIC OXIDE AS A FUNCTION OF CHLORIDE ION CONCENTRATION (MEASURED UNDER A NITROGEN ATMOSPHERE)
Open symbols, 0.31 mM (55 ppm) ascorbate; solid symbols, 3.13 mM (550 ppm) ascorbate. [], [], pH 5.4; 0,0,pH 5.8; Δ, Δ, pH 6.2

Since the concentrations of the acid forms of nitrite and ascorbate at pH 5.45 were about five times those at pH 6.2, the rate of total reaction at the lower pH would have been expected to be twenty-five times that at the higher pH. As an examination of Table 1 shows, such was not the case; for any given concentration of chloride ions, the rates at pH 5.45 were only five times or less than those at pH 6.2. The explanation is that either the ascorbate ion or the nitrite ion was reacting with the acid form of the other species in the nitrous acid reaction. Since it has been reported that the ascorbate ion is more easily oxidized than the acid form (Dahn *et al.* 1960), the reaction was probably between ascorbate ion and nitrous acid.

Practically speaking, within the normal ranges of pH and chloride concentration in meat products, relatively small changes of pH are more effective than large changes of chloride ion concentration. In this study, the reaction of nitrite with 3.13 mM ascorbate went faster at pH 5.4 with no sodium chloride than it did at pH 5.8 with 4% sodium chloride; that is, lowering the pH by 0.4 of a unit was more effective than adding 4% salt. This result suggests that it would require only a relatively small decrease in pH of meat products to compensate for sodium chloride reduction with respect to other nitrite functions such as retarding botulism outgrowth or producing color and flavor.

The Role of Ascorbic Ions

The chloride/nitrite reaction required ascorbate to form nitric oxide; a solution of sodium nitrite, sodium chloride, metmyoglobin and albumin produced no measurable nitric oxide pigment after 24 h. Ascorbate, therefore, is involved in the direct reduction of both nitrous acid and nitrosylchloride to nitric oxide, through the formation of a nitrosated intermediate (Fox and Thomson 1964; Fox and Ackerman 1968). It also has a more generalized role in maintaining reducing conditions in the system whereby nitric oxide is stabilized once formed.

The Effect of Air

The reaction in air was also accelerated by chloride ions (Fig. 2), and the chloride concentration effect was proportionally greater than in nitrogen. In slower reactions at higher pH values than shown in Fig. 2, there was a lag period, probably due to scavenging of oxygen by nitric oxide (Fox *et al.* 1967). Chloride ions contribute nothing toward the total reducing capability of the system, yet the addition of these ions produced more nitric oxide heme pigment in air after 10 h. After 20 h, the solutions with chloride ions faded and had no more red pigment than those without chloride. Both solutions continued to fade until all the pigment reverted to metmyoglobin. In nitrogen, the pigment reached a higher concentration and the color was stable indefinitely with or without the presence of sodium chloride. The conclusion is that for long term stability, exclusion of air is more important to color than the speed with which the color is formed, an effect long recognized in cured meat packaging applications.

In an earlier review (Sebranek and Fox 1986), we postulated the presence of different nitrosating species in mixed media, and pointed out that since they do not all react the same, it is of interest to know to what extent different species are formed in complex mixtures. This study indicates the existence of two different nitrosating species from nitrite in the presence of sodium chloride and sodium ascorbate. Not all nitrosating species are alike in their reactivity, varying not only in the rate of nitrosation, but in the products resulting from different reactants.

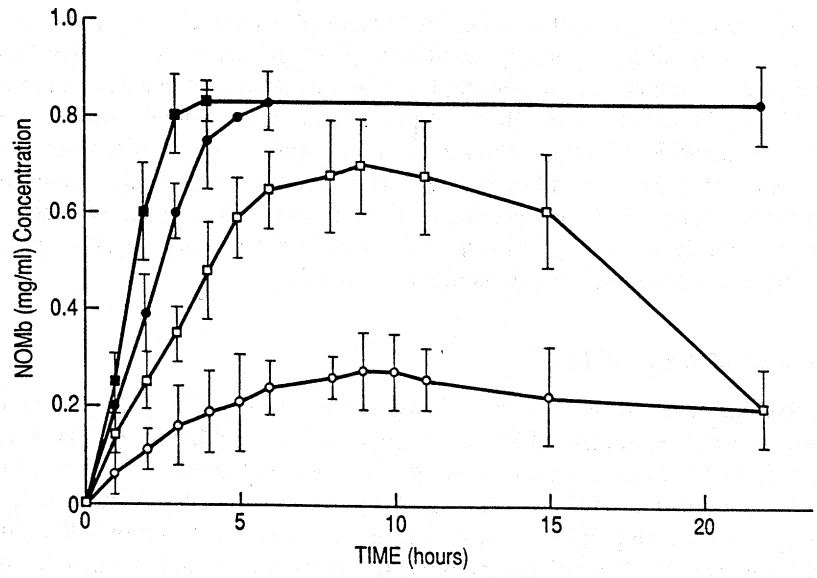

FIG. 2. THE AMOUNT OF NITRIC OXIDE MYOGLOBIN FORMED WITH TIME AT pH 5.4
0, zero percent NaCl; [] 4% NaCl. Solid symbols measured in nitrogen atmosphere, open symbols measured in air

Thus if lowering the chloride ion concentration does not make a very large difference in the amount of cured meat color formed, it might still alter the effectiveness with which nitrite prevents the outgrowth of Clostridia spores or other cured meat characteristics, depending on the active form of nitrite. Since the combination of sodium nitrite and sodium chloride was effective at concentrations where either had little or no effect in spore outgrowth alone (Roberts and Ingram 1973), it appears that nitrosylchloride may be an active agent in preventing outgrowth of botulism spores. Tompkins *et al*, (1978) found that ascorbate enhanced the effect of nitrite in preventing outgrowth, implying that nitric oxide was the reactive species. It appears that nitrosating species are more active in preventing outgrowth than nitrous acid itself, and that some species are more reactive than others.

In conclusion, chloride ions play a role in nitric oxide formation as measured in model systems and, possibly, in meat systems as well. This result is most likely due to the formation of nitrosylchloride, but the practical implications for meat systems are not totally clear. Because nitrosylchloride is likely to be extremely short-lived, we did not intend to directly measure this intermediate. Meat contains many reductants and chemical groups capable or reacting with nitrous acid, nitric oxide, nitrosylchloride, etc. As an example of the problem,

Theiler *et al.* (1981) found that chloride ions suppressed nitrosamine formation, an effect not necessarily expected from an ion that produces a nitrosating species but an effect which may occur if nitrosylchloride transfers nitric oxide to another compound in preference to a secondary amine. These relationships, while based on the observed rates of nitric oxide formation may mean that changing chloride ion concentrations could have several very different effects on cured meat characteristics. Many of these effects may be subtle but, given the importance of sodium nitrite to meat curing, should be considered when reformulating products with reduced levels of sodium chloride.

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